8EHQ-0802-15176

RECEIVED
OPPT CBIC

2002 AUG -2 AM 6:10

National Starch & Chemical
A member of the ICI Group

National Starch and Chemical Company
10 Finderne Avenue
P.O. Box 6500
Bridgewater, New Jersey 08807-0500
908-685-5000

Internet: http://www.nationalstarch.com

Writer's Direct Dial: (908) 707-3756
Fax Number: (908) 685-6955

2002 AUG 29 AM 10:07

July 30, 2002

Document Processing Center (7407)
Attn: TSCA 8(e) Coordinator (Room G99 East Tower)
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460-0001

COMPANY SANITIZED

Re: Modifed Acrylic Terpolymer

To Whom It May Concern:

This letter will serve as a TSCA 8(e) Notification for a potential increased risk to the environment regarding modifed acrylic terpolymer (Confidential Business Information attached). An acute algal toxicity study was conducted on the aforementioned material. The results showed that the above material was toxic to algae. A final report is attached. Additional studies are being conducted to further investigate the behavior of this material in aquatic systems. We will forward this information to you upon completion.

If you have any questions please contact me.

8EHQ-02-15176
88020000 1625

Sincerely,

Janet C. Gould, Ph.D., D.A.B.T.
Senior Toxicologist
Product Assurance and Regulatory Affairs

JCG/
Attachments
cc: P. Mudge

60879

CONFIDENTIAL BUSINESS INFORMATION

MR #
60879

STUDY TITLE

Unicellular Green Alga, *Selenastrum capricornutum*

DATA REQUIREMENTS

OECD Guideline 201

AUTHOR

Stephen L. Hicks

STUDY INITIATION DATE

April 4, 2002

STUDY COMPLETION DATE

May 8, 2002

SPONSOR

National Starch and Chemical Company
10 Finderne Avenue
Bridgewater, New Jersey 08807

PERFORMING LABORATORY

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

PROJECT IDENTIFICATION

ABC Study No. 47368

STATEMENT OF GLP COMPLIANCE

Compliance Statement for ABC Study No. 47368 entitled, '[illegible] to the Unicellular Green Alga, *Selenastrum capricornutum*," for National Starch and Chemical Company, Bridgewater, New Jersey.

The Study Director for the above-referenced test herein confirms that the study was conducted in compliance with the OECD Principles of Good Laboratory Practice (1) with the following exception: the test substance was not characterized in accordance with Good Laboratory Practices by the study Sponsor.

All original raw data were submitted to the Sponsor along with the final report. A copy of the final report, copies of all raw data from the study, and all original facility records are kept on file in ABC Laboratories' archives.

Stephen L. Hicks

Stephen L. Hicks
Study Director
ABC Laboratories, Inc.

08MAY02
Date

QUALITY ASSURANCE STATEMENT

ABC's Quality Assurance Unit reviewed ABC Study No. 47368 entitled, "Toxicity of the Unicellular Green Alga, *Selenastrum capricornutum*," for National Starch and Chemical Company, Bridgewater, New Jersey. The following audits/inspections were conducted on this study.

Date of Inspection	Phase Inspected	Date Reported to the Study Director	Date Reported to ABC Management
18 Apr 02	72-Hour Water Chemistry	18 Apr 02	19 Apr 02
26 Apr 02	Day–3 Water Quality	26 Apr 02	26 Apr 02
03 May 02	Draft Report/Raw Data	03 May 02	06 May 02
08 May 02	Final Report	08 May 02	08 May 02

These audits indicate that the report is an accurate reflection of the study as it was conducted by ABC Laboratories, Inc., and that the protocol and appropriate SOP's were followed.

Jessica Dutton
Quality Assurance Officer I

08 May 02
Date

STUDY PERSONNEL

NAME	TITLE
Stephen L. Hicks	Research Scientist
Andrew Nold	Technical Scientist

SIGNATURE PAGE

Submitted by:

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

Prepared by:

Stephen L. Hicks
Research Scientist

08 MAY 02
Date

Approved by:

Del O. Zoch for
G. Scott Ward
Vice President
Chemical Development Group

8 May 02
Date

TABLE OF CONTENTS

Page No.

COMPENDIUM

Study Title:	to the Unicellular Green Alga, *Selenastrum capricornutum*
Test Substance:	
Nominal Test Concentrations:	0.0 (control), 0.13, 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L
Test Medium:	Freshwater Algal Nutrient Medium at pH 7.5 ± 0.1
Experimental Dates:	Start – April 23, 2002 (definitive test initiation) Termination – April 26, 2002 (definitive test termination)
Length of Study:	72 hours
Temperature Range for Definitive Test Solutions:	24.2 to 25.4°C
pH Range for Definitive Test Solutions:	7.42 to 8.74
Results Based on Nominal Concentrations:	72-Hr E_bC_{50} = 0.30 mg/L (95% confidence limits: 0.28 to 0.33 mg/L) 72-Hr E_rC_{50} = 1.2 mg/L (95% confidence limits: 0.82 to 1.5 mg/L) 72-Hr NOEC = 0.13 mg/L

1.0 INTRODUCTION

National Starch and Chemical Company contracted ABC Laboratories, Inc., to conduct a 72-hour toxicity test with *Selenastrum capricornutum* exposed to The criterion for effect was inhibition in cell growth. Results of the test are expressed as E_bC_{50} and E_rC_{50} concentrations of , calculated to reduce biomass and growth rate, respectively, by 50 percent at the specified time.

2.0 MATERIALS AND METHODS

2.1 Test Substance

A sample of was received from / on March 12, 2002, and was assigned ABC reference no. TS-14270. The sample was stored at room temperature and was used to prepare exposure solutions during the range-finding and definitive tests. The following test substance information was received from the Sponsor:

Lot No.:	91789
Physical Description:	Milky white aqueous emulsion with a mild acrylate odor
Relative viscosity:	24 cps (Brookfield)
Purity (% solids):	21.84%
Density:	1.04 g/cm^3
pH:	8.19
Expiration Date:	March 2003
Storage Information:	Ambient temperature

2.2 Test Organism

The parent stock of *Selenastrum capricornutum* (UTEX no. 1648) was obtained from the Department of Botany, Culture Collection of Algae, University of Texas at Austin, on November 20, 2001. The algal culture was identified by the supplier as *Selenastrum capricornutum*. The parent culture was used to prepare individual cultures by transferring portions of the parent culture to culture flasks containing medium. The prepared cultures were incubated at 24 ± 2°C in an environmental chamber under continuous fluorescent lighting. Periodically, new *Selenastrum capricornutum* cultures were initiated using the parent stock or were cloned from an existing culture derived from the parent stock in 100 mL of algal nutrient medium. All cultures were maintained under the same conditions as those used for testing. The algal culture used for the definitive test was seven days old at test initiation.

2.3 Test Medium

The test medium was a freshwater algal nutrient medium prepared by addition of reagent grade salts to ABC reagent water (2). After preparation, the medium was filtered through Millipore® 0.45-µm filters. Chemical characterization of a representative sample of ABC reagent water is presented in Appendix A.

2.4 Test Methods

Test procedures followed the ABC test protocol entitled "Toxicity of to the Unicellular Green Alga, *Selenastrum capricornutum*," and amendments (Appendix B). This protocol was designed to meet Organization of Economic Cooperation and Development method 201 (3).

2.4.1 Range-Finding Test

A range-finding test was conducted from April 5 to 8, 2002, at nominal test concentrations of 0 (control), 0.10, 1.0, 10, 100, and 1000 mg/L. After 72 hours of exposure, percent decrease in cell density, as compared to the control, ranged from 0% in the 0.10-mg/L treatment to 100% in the 100 and 1000-mg/L treatments.

An unsuccessful definitive test was conducted from April 15 to 18, 2002, and was used as additional range-finding test data since the 72-hour no-observed effect concentration was estimated to be less than the lowest concentration tested. The test was conducted at nominal test concentrations of 0 (control), 0.25, 0.50, 2.0, 4.0, and 8.0 mg/L. After 72 hours of exposure, percent decrease in cell density, as compared to the control, ranged from 11% in the 0.25-mg/L treatment to 100% in the 8.0-mg/L treatment. Based upon the results of this test, nominal concentrations of 0 (control), 0.13, 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L were selected for the definitive test.

2.4.2 Definitive Test

The definitive test was conducted from April 23 to 26, 2002. The definitive test was performed in autoclaved 250-mL Erlenmeyer flasks fitted with foam stoppers. The control and each treatment were replicated three times. The control contained no test substance. Each flask was labeled with the ABC study number, concentration, replicate, and grid position. The flasks were randomly positioned on a orbital shaker, set at approximately 100 rpm, and were incubated at 24 ± 2°C for 72 hours in a temperature controlled environmental chamber. Continuous cool-white fluorescent lighting was provided with a light intensity of 8600 ± 10% lux. The light intensity was measured with a LI-COR Model LI-189 light meter equipped with a photometric sensor.

A 0.10-mg/mL primary standard was prepared by diluting 0.2293 g (0.0501 g corrected for purity) of a 500-mL volume with freshwater algal media. A 0.0040-mg/mL working standard was prepared by diluting 40 mL of the 0.10-mg/mL primary standard to a 1-L volume with freshwater algal media. This working standard was used for the highest treatment and the five lower treatments were prepared individually using appropriate volumes of the working standard and freshwater algal media.

The definitive test was conducted for 72 hours commencing when the flasks were inoculated with *Selenastrum capricornutum*. Each flask was inoculated with 1.0 mL of a *Selenastrum capricornutum* inoculum containing approximately 1.0×10^6 cells/mL, resulting in an initial cell

density of approximately 1.0×10^4 cells/mL. Cell counts were performed using a light microscope and a hemacytometer for each control and test substance treatment replicate once every 24 hours.

Temperature and pH were measured in each parent solution at 0 hour of the test. Temperature and pH were measured in one replicate of the control and each test substance treatment at 72 hours of the test. Temperature and pH were measured with a Denver Instruments pH meter. A continuous recording of the enclosure temperature was made using a datalogger and thermistor probe.

2.5 Analytical Confirmation

No analytical confirmation was performed to measure the test concentrations of during the range-finding or definitive tests.

2.6 Statistical Analysis

The 24-, 48-, and 72-hour EC and NOEC values were calculated, if data allowed calculation, using SAS (4) and were based on area under the growth curve (E_bC) and growth rate (E_rC) versus the nominal concentrations of . Prior to the EC and NOEC calculations, a Shapiro-Wilk's test (5) and a Levene's test (6) were conducted to test for normality and homogeneity of variance, respectively, over treatments at each time point. Where *p* values from the Shapiro-Wilk's and Levene's test were greater than 0.01, indicating normality and insignificant heterogeneity, the analysis was performed on the non-transformed raw data. Where *p* values were less than 0.01 for some hour(s), the raw data for each replicate were transformed using the square root of the raw data, the recommended transformation for count data (7). When the raw data and transformed data displayed nonnormality or inequality of variance, a nonparametric analysis of variance was performed using the ranks of the values (5). Non-parametric tests were performed on all data.

A one-way analysis of variance (ANOVA) and a Dunnett's comparison to the control was conducted for each time point to determine the NOEC's. A one-tailed Dunnett's test was conducted at the 0.05 level of significance with the alternate hypothesis being that the area under the growth curve, and/or growth rate had been reduced in comparison to the control.

For the control and each test substance treatment group, the area under the growth curve was calculated by SAS from time 0 to each observation period using the following equation:

$$A = \frac{N_1 - N_0}{2} x\, t_1 + \frac{N_1 + N_2 - 2N_0}{2} x\left[t_2 - t_1\right] + \ldots + \frac{N_{n-1} + N_n - 2N_0}{2} x\left[t_n - t_{n-1}\right]$$

where:

A	=	area
N_n	=	cell density at n^{th} measurement from start, n=1,2,...n
N_o	=	cell density at time 0
t_n	=	time of n^{th} measurement (hours after start), n=1,2,...n

For the control and each test substance treatment group, the growth rate was calculated by SAS for each time interval using the following equation:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

where:

μ	=	growth rate
N_n	=	cell density at second time point
N_1	=	cell density at time 0
t_n	=	second time point
t_1	=	time 0

The E_bC_{50} and E_rC_{50} values and their 95% confidence limits were calculated by SAS using nonlinear (weighted) regression. The SAS nonlinear modeling procedure developed a logistic (sigmoid-shaped) model which was fit to the data with percent inhibition as the dependent variable and concentration as the independent variable. The percent inhibition was calculated compared to the control based on area under the growth curve and growth rate. The % inhibition was calculated for area under the growth curve and growth rate according to the following formula:

$$\frac{Control\ Mean - Treatment}{Control\ Mean} \times 100 = \%\ Inhibition$$

3.0 RESULTS AND DISCUSSION

3.1 Test Solutions

The control and all test solutions were clear and colorless with no visible precipitate or surface film at test initiation. The control and treatments ≤0.25 mg/L were green in color after 48 hours of exposure and the control and all treatements ≤2.0 mg/L were green in color after 72 hours of exposure. The green coloration resulted from an increase in algal biomass. The 4.0-mg/L treatment remained clear and colorless with no visible precipitate or suface film for the duration of the exposure.

3.2 Biological Results

After 72 hours of exposure, the mean number of cells in the control was 125×10^4 cells/mL (Table 1). This value represented an increase of 125 times the initial target inoculation density and demonstrated control growth was acceptable for the test. The mean number of cells at 72 hours ranged from 126×10^4 cells/mL in the 0.13 mg/L treatment to 0.87×10^4 cells/mL in the 4.0-mg/L treatment (Table 1). Percent inhibition in algal growth (cell density) ranged from 0% in the 0.13-mg/L treatment to 99% in the 4.0-mg/L treatment. Cell counts for all replicates are presented in Table 2. Growth curves for the control and all treatments are

presented in Figure 1. Mean area under the growth curve and mean growth rate values determined by SAS are presented in Tables 3 and 4, respectively. Individual replicate values for areas under the growth curve and growth rate are presented in appendices C and D, respectively. No significant reduction in algal growth was detected in the 0.13-mg/L treatment as measured by area under the growth curve and growth rate after 72 hours of exposure to [redacted]. The 72-hour E_bC_{50} and E_rC_{50} values were 0.30 mg/L (95% confidence limits: 0.28 to 0.33 mg/L) and 1.2 mg/L (95% confidence limits: 0.82 to 1.5 mg/L), respectively (Table 5). The 72-hour NOEC was 0.13 mg/L. All results were based on the nominal concentrations.

3.3 Water Quality

Test solution temperature ranged from 24.2 to 25.4°C at 0 and 72 hours (Table 5). The temperature data from the datalogger indicated that the temperature of the environmental chamber during the definitive test remained within the 24 ± 2°C range specified in the protocol. The pH of the control solution was 7.47 at test initiation and the pH of all treatment solutions ranged from 7.42 in the 2.0- and 4.0-mg/L treatments to 7.47 in the 0.50-mg/L treatment. (Table 6). The pH at 72 hours remained between 7.65 in the 4.0-mg/L treatment to 8.74 in the 0.13-mg/L treatment (Table 6). The pH of the control and treatments ≤0.25 mg/L deviated more than 1 pH unit from the 0-hour to the 72-hour measurements which was a result of the algal biomass present at 72 hours. The pH deviation of more than 1 pH unit did not affect the integrity of the test since acceptable growth (>16X increase) was observed in the controls.

4.0 CONCLUSIONS

The 72-hour E_bC_{50} and E_rC_{50} values were 0.30 mg/L (95% confidence limits: 0.28 to 0.33 mg/L) and 1.2 mg/L (95% confidence limits: 0.82 to 1.5 mg/L), respectively. The 72-hour NOEC was 0.13 mg/L. All results were based on the nominal concentrations.

REFERENCES

(1) Organization for Economic Cooperation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

(2) American Society for Testing and Materials (ASTM). 1997. Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM Designation E1218-97a.

(3) Organization for Economic Cooperation and Development (OECD). June 7, 1984. OECD Guidelines for Testing of Chemicals. Algae, Growth Inhibition Test, OECD Guideline No. 201.

(4) The SAS System for Windows, Release 6.12. Copyright 1989-1996 by SAS Institute Inc., Cary, North Carolina, 27513, USA.

(5) Conover, W.J. *Practical Nonparametric Statistics*. 1980. 2nd Ed.

(6) Milliken, G.A. and D.E. Johnson. *Analysis of Messy Data*. 1984. Vol. 1. p. 22.

(7) Zar, J.H. *Biostatistical Analysis*. 1984. 2nd Ed. p. 241.

Table 1. Mean Cell Numbers of the Green Alga, *Selenastrum capricornutum*, Exposed to

Nominal Concentration (mg/L)	Mean Cell Numbers (× 10^4 cells/mL)[a]			Percent Inhibition[b]
	24 Hr	48 Hr	72 Hr	
Control	4.1	22	125	---
0.13	2.6	20	126	0
0.25	0.96	7.8	90	28
0.50	0.037	1.4	44	65
1.0	0	0.26	13	90
2.0	0	0.15	6.0	95
4.0	0	0	0.87	99

[a] Values are means of triplicate test chambers.

[b] Percent inhibition in cell density at 72 hours compared to the control value of 125 x 10^4 cells/mL at 72 hours.

NOTE: The target cell density at test initiation was 1.0 x 10^4 cells/mL.

Table 2. Replicate Cell Numbers for the Green Alga, *Selenastrum capricornutum*, During a 72-Hour Exposure to

Nominal Concentration (mg/L)	Rep	Cell Numbers ($\times 10^4$ cells/mL)		
		24-Hour	48-Hour	72-Hour
Control	A	3.6	21	125
	B	4.4	23	127
	C	4.4	23	123
0.13	A	2.7	22	131
	B	2.3	20	125
	C	2.7	19	123
0.25	A	1.0	11	103
	B	1.0	7.4	81
	C	0.89	5.1	86
0.50	A	0	2.0	48
	B	0.11	0.89	41
	C	0	1.2	42
1.0	A	0	0.33	7.8
	B	0	0.22	14
	C	0	0.22	18
2.0	A	0	0.22	5.1
	B	0	0.22	8.8
	C	0	0	4.1
4.0	A	0	0	1.6
	B	0	0	0.78
	C	0	0	0.22

Note: All cells observed were normal in appearance.

Table 3. Mean Area Under the Growth Curve Values for the Green Alga, *Selenastrum capricornutum*, Exposed to

Nominal Concentration (mg/L)	Mean Area Under Growth Curve[a]			Percent Inhibition[b]
	0-24 Hr	0-48 Hr	0-72 Hr	
Control	38	330	2,100	---
0.13	19[c]	270[c]	2,000	5
0.25	-0.44[c]	81[c]	1,200[c]	43
0.50	-12[c]	-19[c]	500[c]	76
1.0	-12[c]	-33[c]	110[c]	95
2.0	-12[c]	-34[c]	16[c]	99
4.0	-12[c]	-36[c]	-50[c]	102

[a] Values determined by SAS are means of triplicate test chambers and rounded to two significant figures.

[b] Percent inhibition in area under the growth curve at 72 hours compared to the control value of 2,100 at 72 hours.

[c] Area under the growth curve was significantly ($p = 0.05$) less when compared to the control.

Table 4. Mean Growth Rate Values for the Green Alga, *Selenastrum capricornutum*, Exposed to

Nominal Concentration (mg/L)	Mean Growth Rate (cells/mL/hour)[a]			Percent Inhibition[b]
	0-24 Hr	0-48 Hr	0-72 Hr	
Control	0.059	0.065	0.067	--
0.13	0.039[c]	0.063	0.067	0
0.25	-0.0016[c]	0.042[c]	0.062[c]	7
0.50	-0.11[c]	0.0053[c]	0.052[c]	22
1.0	-0.12[c]	-0.029[c]	0.035[c]	48
2.0	-0.12[c]	-0.041[c]	0.024[c]	64
4.0	-0.12[c]	-0.060[c]	-0.0060[c]	109

[a] Values determined by SAS are means of triplicate test chambers and rounded to two significant figures.
[b] Percent inhibition in growth rate at 72 hours compared to the control value of 0.067 at 72 hours.
[c] Area under the growth curve was significantly ($p = 0.05$) less when compared to the control.

Table 5. Calculated EC Values Based on Nominal Concentrations of During an Exposure of the Green Alga, *Selenastrum capricornutum*

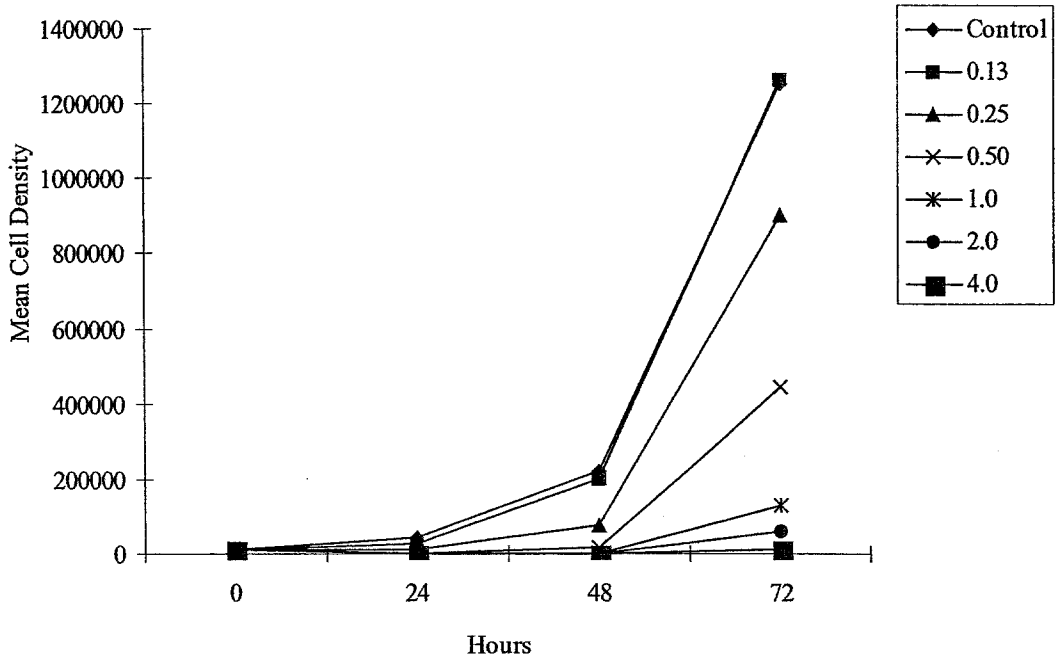
	Effect Concentration Expressed as mg/L	
Time Point	E_bC_{50} (95% Confidence Limits)	E_rC_{50} (95% Confidence Limits)
24-hour	<0.13	Could not be calculated
48-hour	0.20 (0.15 – 0.24)	0.28 (0.021 – 0.55)
72-hour	0.30 (0.28 – 0.33)	1.2 (0.82 – 1.5)

Table 6. Temperature and pH Measurements During the Exposure of *Selenastrum capricornutum* to

Nominal Concentration (mg/L)	0-Hour[a]		72-Hour[b]	
	Temp (°C)	pH	Temp (°C)	pH
Control	24.8	7.47	24.8	8.65
0.13	24.3	7.45	25.4	8.74
0.25	24.2	7.46	24.9	8.52
0.50	24.3	7.47	24.2	8.11
1.0	24.2	7.46	24.6	7.85
2.0	24.4	7.42	25.0	7.67
4.0	24.2	7.42	25.0	7.65

[a] Measured in parent solutions.
[b] Measured in replicate A.

Figure 1. Growth Curves for the Green Alga, *Selenastrum capricornutum*, During a 72-Hour Exposure to



APPENDIX A. ABC REAGENT WATER CHARACTERIZATION

January 2001 ABC Reagent Water Screen

Chlorinated Hydrocarbons (μg/L)	2001	Historical Range 98-01
DDE	<0.040	<0.040
DDD	<0.040	<0.040
DDT	<0.040	<0.040
Dieldrin	<0.040	<0.040
α-BHC	<0.040	<0.040
β-BHC	<0.040	<0.040
γ-BHC	<0.040	<0.040
Δ-BHC	<0.040	<0.040
Heptachlor Epoxide	<0.040	<0.040
Endrin	<0.040	<0.040
Methoxychlor	<0.040	<0.095
Toxaphene	<0.10	<3.8
Chlordane	<0.10	<0.47
Endosulfan I	<0.040	<0.040
Endosulfan II	<0.040	<0.040
Endosulfan Sulfate	<0.040	<0.040
Aroclor 1016	<0.10	<0.10
Aroclor 1221	<0.10	<0.10
Aroclor 1248	<0.10	<0.10
Aroclor 1232	<0.10	<0.10
Aroclor 1242	<0.10	<0.10
Aroclor 1254	<0.10	<0.10
Aroclor 1260	<0.10	<0.10

Elements (mg/L)	2001	Historical Range 98-01
Arsenic	<0.010	<0.010
Boron	<0.020	<0.020
Cadmium	<0.0050	<0.0050
Chromium	<0.010	<0.010
Copper	<0.010	<0.010
Iron	<0.10	<0.10
Lead	<0.0050	<0.0065
Mercury	<0.00020	<0.00050
Nickel	<0.020	<0.020
Selenium	<0.0050	<0.0050
Silver	<0.0050	<0.0050
Zinc	<0.020	<0.020

Organophosphate (μg/L)		
Diazinon	<1.0	<1.0
Parathion	<1.0	<1.0
Malathion	<1.0	<1.0
Ethion	<1.0	<1.0
Disulfoton	<1.0	<1.0
Azinphos Methyl	<1.0	<1.0
Demeton, Total	<1.0	<1.0

Miscellaneous (mg/L)		
Nitrite N	<0.050	<0.050
Nitrate N	<0.050	<0.050-0.17
Total Phosphorus as P	<0.010	<0.010-0.60

Note: Data supporting these values are on file at ABC Laboratories.

APPENDIX B. PROTOCOL AND AMENDMENTS

Toxicity of to the Unicellular Green Alga, *Selenastrum capricornutum*

ABC Study No. 47368

This protocol complies with
OECD Guideline 201

This protocol is based upon ABC generic protocol E101

1.0 STUDY TITLE

Toxicity of to the Unicellular Green Alga, *Selenastrum capricornutum*

2.0 OBJECTIVE

The primary objective of this test is to determine the 72-hour EC_{50} (E_bC_{50} and/or E_rC_{50}) of the test substance to the unicellular green alga, *S. capricornutum*, under static test conditions. An EC_{50} (E_bC_{50} and/or E_rC_{50}) is the approximate concentration of the test substance that inhibits 50% of algal growth (measured as biomass or growth rate), relative to the control. In addition, the no-observed-effect concentration (NOEC) at 72 hours will be determined, if possible.

3.0 STUDY SPONSOR

National Starch and Chemical Company
10 Finderne Avenue, P.O. Box 6500
Bridgewater, New Jersey 08807-0500
TEL: (908) 707-3756 FAX: (908) 685-6955
Sponsor Representative: Janet C. Gould, Ph.D.

4.0 TESTING FACILITY AND STUDY DIRECTOR ADDRESS

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202
TEL: (573) 474-8579 FAX: (573) 443-9089
Study Director: Steve Hicks

5.0 PROPOSED SCHEDULE

PROPOSED EXPERIMENTAL START DATE: April 2002
PROPOSED EXPERIMENTAL COMPLETION DATE: May 2002

6.0 TEST PROTOCOL

The test protocol which follows is based on method 201 of the OECD Guidelines for Testing of Chemicals (1).

7.0 TEST AND REFERENCE SUBSTANCES

7.1 Test Substance

The test substance will be The sample, a milky white aqueous emulsion with a mild acrylate odor, has a purity of 21.84% (% solids), relative viscosity of 24 cps (Brookfield), density of 1.04 g/cm^3, pH of 8.19, and expiration date of March 2003. The sample should be stored at ambient temperature.

7.2 Reference Substance

There will be no reference substance (i.e., analytical standard) used since analytical confirmation of test substance solutions will not be performed.

7.3 Sample Characterization and Retention

Characterization, stability, and solubility studies will be the responsibility of the Sponsor unless otherwise contracted to ABC Laboratories, Inc. The test and reference substances will be returned to the Sponsor following completion of their use at ABC Laboratories, Inc. Archival of retention samples will also be the Sponsor's responsibility.

7.4 Test Substance Preparation/Addition

The test concentrations will be prepared on a weight/volume basis unless otherwise specified. Test concentrations will be based on a percent solids basis. If toxicity is not observed at or below the maximum solubility of the test substance in the test medium, the highest test concentration definitively tested will be the maximum solubility of the test substance in the test medium or 1000 milligrams per liter (mg/L), whichever is lower.

8.0 TEST SYSTEM

8.1 Species

The unicellular green alga, *Selenastrum capricornutum*. The species will be initially identified by the supplier and then periodically inspected to insure the purity of the culture.

8.2 Justification

When freshwater algal toxicity data are generated following OECD guideline 201, *Selenastrum capricornutum* is one of the recommended species.

8.3 Source

S. capricornutum (UTEX 1648) will be obtained from an established ABC Laboratories' culture which originated with an inoculum received from the University of Texas, Austin, Texas.

8.4 Age

Transfers will be made regularly into fresh algal nutrient medium to provide 5- to 10-day old cultures, or cultures with sufficient cell density for test inoculations.

8.5 Culture

The algae will be cultured in freshwater algal nutrient medium under continuous illumination of approximately 8600 lux at a temperature of 24 ± 2°C.

9.0 TEST MEDIUM

The test medium will be filtered (0.45 micrometers) freshwater algal growth medium prepared with ABC reagent water and reagent grade chemicals (2). The pH of the medium prior to inoculation will be 7.5 ± 0.1 and may be adjusted to this pH with 0.1N NaOH or HCl prior to test substance addition. Depending upon the situation, the pH may be adjusted following test substance addition, but prior to inoculation of algae. ABC reagent water used in the preparation of the algal growth medium is chemically characterized annually per ABC SOP to verify that it is free of contaminants which might interfere with test results.

10.0 TEST PROCEDURES

Generally two toxicity tests will be conducted, a range-finding and a definitive test. The range-finding test is an abbreviated toxicity test employing widely spaced test concentrations to define the approximate range within which the test substance produces a gradient from nontoxic to toxic effects. The range-finding test is conducted using the same basic procedures and conditions as those used during definitive tests. Results of the range-finding test(s) guide selection of the test concentrations for the definitive test, the purpose of which is provide a precise estimate of the 72-hour median effective concentration (EC_{50}) of the test substance which affects the growth of this alga.

10.1 Range-Finding Test

The range-finding test(s) will be initiated by inoculating at least one flask per test substance concentration with a predetermined aliquot of algal inoculum. The test concentrations will typically cover several orders of magnitude (e.g., 1.0, 10, 100, 1000 mg/L). Typically, test condition parameters such as light intensity, oscillation rate, test solution pH and temperature will be measured at test initiation and termination. Additional or fewer measurements may be made at the discretion of the Study Director. At a minimum, cell counts will be determined after approximately 72 hours of exposure.

10.2 Definitive Test

10.2.1 Experimental Design

The definitive test will consist of one or more control treatments and a geometric series (ratio between concentrations ≤2.2) of at least five test substance concentrations. If any vehicle other than test medium or water is present in any of the test vessels, a vehicle control will be maintained concurrently. The vehicle control will possess the greatest concentration of vehicle present in any of the treatments. If a vehicle is utilized, the concentration of vehicle will not exceed 100 µL/L. Definitive test concentrations and vehicle control, if needed, will be specified by protocol amendment. All test chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, vehicle control, level 1, level 2, etc.), replicate (e.g., A, B, etc.) and grid position.

The definitive test will generally be conducted in 250-mL Erlenmeyer flasks fitted with foam stoppers to permit gas exchange and to prevent contamination. If 250-mL flasks are used, each flask will contain 100 mL of test solution. The size of the Erlenmeyer flasks is not critical, but the sample-to-volume ratio should not exceed 50%. Flasks used in testing will be cleaned and sterilized (autoclaved) according to the ABC Laboratories' SOP. Three replicates will be used for each control, vehicle control (if necessary), and test substance concentration. Each replicate will be inoculated with algae and placed on a rotary shaker at approximately 100 revolutions per minute. For the control of bias among replicates, test flasks used during the definitive test will be assigned to the testing area using a computer-generated randomization table. The algal inoculum will be from a 5-10-day-old stock culture or a culture with sufficient cell density to yield a final inoculum

density of 1×10^6 cells/mL. The algal cells will be rinsed of the culture medium and then resuspended in test medium. The test algae will be inoculated into the test flasks within 30 minutes after preparation of test solutions to yield an approximate initial cell density of 1×10^4 cells/mL. The test flasks will then be incubated in a temperature-controlled enclosure illuminated continuously for 72 hours. This route of administration was selected to comply with OECD guideline 201.

A limit test may be performed at a concentration of 1000 mg/L or at a concentration equal to the solubility of the substance in the medium for situations in which the algal growth inhibition is estimated to be less than 50% at this maximum test concentration. The limit test will be performed in triplicate, with the same number of controls. If, in a limit test, a mean decrease of 25% or more is found in either biomass or growth rate between the limit test and the control, a full test should be carried out.

10.2.2 Lighting and Oscillation

Throughout the test, the test flasks will be illuminated continuously by cool-white fluorescent bulbs that provide 8600 ± 860 lux and continuously swirled on an orbital shaker table at approximately 100 rpm. Daily light readings (measured at the level of the test solutions) and shaker oscillation rate will be measured and recorded.

10.2.3 Chemical/Physical Parameters

Temperature of the environmental chamber will be measured continuously during the definitive test. Temperature and pH will be measured at 0 and 72 hours in the control, vehicle control (if necessary) and all test substance concentrations. Measurement at test initiation (0-hour) will be conducted on all parent solutions (prior to distribution of the solutions to the test vessels). Measurements at test termination will be conducted on at least one replicate of the control, vehicle control (if necessary), and all test substance concentrations. The temperature should be 24 ± 2°C and the pH of the solutions should not normally deviate more than 1 pH unit during the test.

10.2.4 Biological Data

Cell density will be determined for each replicate of the control, vehicle control (if necessary), and each test concentration at 24, 48, and 72 hours (±1 hour from test initiation) to evaluate algal growth (inhibition or

enhancement). Cell density may also be determined at 0 hour for each replicate of the control and vehicle control (if necessary) to confirm initial cell densities. Cell density determinations will be accomplished using a hemacytometer and an optical microscope. In addition to cell density determinations, microscopic examination will be conducted to determine if there are any morphological or physical effects on the algal cells. Unusual cell shapes, color differences, flocculation, adherence of algae to test chambers, or aggregation of algal cells will be noted.

10.2.5 Analytical Confirmation

No analytical confirmation of test solutions will be conducted.

11.0 ANALYSIS OF RESULTS

The results of the definitive test will be examined to determine those concentrations that inhibit or enhance growth of the test algae. Generally, results will be reported using the overall mean measured concentrations (mean of 0- and 72-hour measured concentrations) when test solutions have been analyzed, unless otherwise requested by the study sponsor. When test solutions have not been analyzed, results will be reported using the nominal concentrations.

The results of the definitive test will be statistically analyzed for 24-, 48-, and 72-hour EC_{50} (E_bC_{50} and/or E_rC_{50}) values and corresponding 95% confidence limits, if data permit. These values will be determined by the SAS nonlinear modeling procedure (four parameter logistic model with two parameters fixed). The method used will be identified in the report. In addition, the no-observed-effect concentration (NOEC) for at least 72 hours will be determined, if possible, by a one-way analysis of variance (ANOVA) and a multiple means comparison test using the individual replicates values of the areas under the growth curves or the specific growth rates. Additional effect concentrations can be estimated, if desired.

12.0 TEST ACCEPTABILITY CRITERIA

The number of algal cells in the control(s) at test termination should be at least 16 times the number initially inoculated to verify logarithmic phase growth. Unless the maximum test concentration of test substance is tested (i.e., at maximum solubility or 1,000 mg/L), one test concentration should exhibit ≤50% decrease in growth and one concentration should exhibit ≥50% decrease in growth relative to the control(s).

13.0 REPORT

A final report will be submitted to the Sponsor and will include, but not be limited to, the following:

- Study dates, name, and address of test facility.
- Objectives and test procedures as stated in approved protocol.
- A description of the experimental design along with a description of and reference to any statistical methods used for data analysis.
- Description of test substance (e.g., date of receipt, storage conditions, method of preparing stock and/or test solution and, if available, purity, physical characteristics, water and organic solvent solubility) and identification of the reference substance, if applicable.
- Description of test conditions during the study (e.g., vehicle used, dilution water, test temperature, lighting, and pH).
- Description of methods used during the study.
- Description of test system (e.g., source, culture techniques, etc.).
- Summary of the data and a statement of the conclusions drawn from any data analyses, if appropriate.
- Location of raw data.
- List of all study personnel.
- GLP compliance statement by the Study Director and a statement by ABC Laboratories' Quality Assurance Unit.

14.0 PROTOCOL AMENDMENTS AND DEVIATIONS

The Study Director, upon approval of the Sponsor Representative, may make amendments to this protocol. All amendments will describe the change(s), the reason(s) for the amendment, and the effect on the study, if any. All amendments will be signed and dated by at least the Study Director and the Sponsor Representative, and maintained with the protocol.

In the event of a protocol deviation, a written description of the deviation, including the reason for the deviation and any impact on the study as a result of the deviation, will be submitted to the Sponsor Representative. All deviations will be signed and dated by at least the Study Director and the Sponsor Representative.

15.0 QUALITY ASSURANCE

ABC's Quality Assurance Unit will inspect one or more critical phases to assure that equipment, personnel, procedures, and records conform to the guidelines listed in this protocol. The results of these inspections will be reported to the Study Director and ABC management. The draft and final reports will be reviewed for protocol and GLP compliance, as well as to assure that the methods and standard operating procedures used were followed. A signed statement will be included in the report specifying types of inspections made, the dates inspections were made, and the dates inspections were reported to the Study Director and management.

16.0 GLP COMPLIANCE

This study will be conducted in accordance with OECD Principles of Good Laboratory Practice (3). The report will contain a statement attesting to that fact.

17.0 RECORDS

Records to be maintained will include, but not be limited to, test substance receipt; solution preparations and dilutions; instrument logbooks detailing calibration and maintenance; facility records (kept at ABC); material control identification numbers for all instruments used; storage of test substance, solutions, and samples; and weights and volumes. All original raw data collected during this study will be maintained at ABC Laboratories until finalization of the study. Upon completion of the study, all original raw data will be submitted to the Sponsor along with the final report. A copy of the final report, copies of all raw data from the study, and all original facility records will be kept on file in ABC Laboratories' archives.

18.0 REFERENCES

(1) Organization for Economic Cooperation and Development (OECD). June 7, 1984. OECD Guidelines for Testing of Chemicals. Algae, Growth Inhibition Test, OECD Guideline No. 201.

(2) American Society for Testing and Materials (ASTM). 1997. Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM Designation E1218-97a, 14 pp.

(3) Organization for Economic Cooperation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

PROTOCOL APPROVAL

ABC Laboratories' Study Director

Name (signed): Stephen L. Hicks Date: 04 APR 02

Name/Title: Steve Hicks/Research Scientist

Sponsor Representative

Name (signed): Date: 03-27-02

Name/Title: Janet C. Gould, Ph.D./Industrial Toxicologist

ABC Laboratories' QAU Protocol Review for GLP Compliance

Name (signed): Jill Yager Date: 04 Apr 02

Name/Title: Jill Yager/QA Officer I

Test Facility Management

Name (signed): G. Scott Ward Date: 4 April 02

Name/Title: G. Scott Ward/Vice President, Chemical Development Group

PROTOCOL ALTERATION NOTIFICATION

PROTOCOL TITLE: Toxicity of . to the Unicellular Green Alga, *Selenastrum capricornutum*

LABORATORY: ABC Laboratories, Inc. **SPONSOR:** National Starch and Chemical Company

ABC STUDY NO.: 47368
ALTERATION NO.: 1 **EFFECTIVE DATE:** 10 APR 02

AMENDMENT:

1. Protocol Section: 10.2.1 Experimental Design

   The definitive test will be conducted with the following nominal concentrations: 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L.

   Reason: Identify definitive test concentration selected from range-finding test results.

   Effect on Study: None

ABC LABORATORIES' STUDY DIRECTOR'S SIGNATURE: Stephen L. Hicks **DATE:** 19 APR 02

STUDY SPONSOR'S SIGNATURE: **DATE:** 4-11-02

PROTOCOL ALTERATION NOTIFICATION

PROTOCOL TITLE: Toxicity of [redacted] to the Unicellular Green Alga, *Selenastrum capricornutum*

LABORATORY: ABC Laboratories, Inc. **SPONSOR:** National Starch and Chemical Company

ABC STUDY NO.: 47368
ALTERATION NO.: 2 **EFFECTIVE DATE:** 23 APR 02

AMENDMENT:

1. Protocol Section: Alteration No. 1; Amendment No. 1

   The definitive test will be conducted with the following nominal concentrations: 0.13, 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L.

   Reason: Identify definitive test concentration selected from additional range-finder test results. This range was established to determine the no-observed effect concentration.

   Effect on Study: None

ABC LABORATORIES' STUDY DIRECTOR'S SIGNATURE: Stephen L Hicks **DATE:** 30 APR 02

STUDY SPONSOR'S SIGNATURE: [signature] **DATE:** April 23, 2002

APPENDIX C. REPLICATE AREAS UNDER THE GROWTH CURVE

Table C-1. Replicate Areas Under the Growth Curve During a 72-Hour Static Exposure of *Selenastrum capricornutum* to

Nominal Concentration (mg/L)	Rep	Area Under the Growth Curve		
		0-24 Hours	0-48 Hours	0-72 Hours
Control	A	31	300	2000
	B	41	350	2100
	C	41	350	2100
0.13	A	20	290	2100
	B	16	260	2000
	C	20	260	1900
0.25	A	0	120	1500
	B	0	77	1100
	C	-1.3	47	1100
0.50	A	-12	-12	560
	B	-11	-23	460
	C	-12	-22	470
1.0	A	-12	-32	42
	B	-12	-33	110
	C	-12	-33	160
2.0	A	-12	-33	6.5
	B	-12	-33	51
	C	-12	-36	-11
4.0	A	-12	-36	-41
	B	-12	-36	-51
	C	-12	-36	-57

Note: Reported values are rounded to two significant figures.

APPENDIX D. REPLICATE GROWTH RATES

Table D-1. Replicate Growth Rates During a 72-Hour Static Exposure to of *Selenastrum capricornutum* to

Nominal Concentration (mg/L)	Rep	Growth Rate (cells/mL/hour)		
		0-24 Hours	0-48 Hours	0-72 Hours
Control	A	0.053	0.063	0.067
	B	0.062	0.065	0.067
	C	0.062	0.065	0.067
0.13	A	0.041	0.064	0.068
	B	0.035	0.062	0.067
	C	0.041	0.061	0.067
0.25	A	0	0.050	0.064
	B	0	0.042	0.061
	C	-0.0049	0.034	0.062
0.50	A	-0.12	0.014	0.054
	B	-0.092	-0.0024	0.052
	C	-0.12	0.0038	0.052
1.0	A	-0.12	-0.023	0.029
	B	-0.12	-0.032	0.037
	C	-0.12	-0.032	0.040
2.0	A	-0.12	-0.032	0.023
	B	-0.12	-0.032	0.030
	C	-0.12	-0.060	0.020
4.0	A	-0.12	-0.060	0.0065
	B	-0.12	-0.060	-0.0035
	C	-0.12	-0.060	-0.021

Note: Reported values are rounded to two significant figures.